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(54) Title: ASSAY FOR DETECTION OF HIV ANTIGEN AND HIV ANTIBODY

(57) Abstract

An assay to simultaneously detect the presence of HIV antigen analyte and/or HIV-1 antibody and/or HIV-2 antibody in a test sample. The analytes are captured on either the same or different solid phases, and the presence of the analytes is determined by detecting a signal generated by analyte-specific indicator reagents.

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ASSAY FOR DETECTION OF HIV ANTIGEN AND HIV ANTIBODY

This application is a continuation-in-part of U.S.S.N. 787,710 filed November 4, 1991, which is a continuation application of U.S.S.N. 07/361,733 filed June 2, 1989 (abandoned), which is a continuation-in-part application of U.S.S.N. 07/320,882 filed March 9, 1989 (abandoned), which is a continuation application of U.S.S.N. 07/020,282 filed February 27, 1987 (abandoned) which is a continuation-in-part application of U.S.S.N. 06/811,240 filed December 20, 1985 (abandoned), which enjoy common ownership and are incorporated herein in reference.

BACKGROUND OF THE INVENTION

This invention relates generally to immunoassays, and more particularly, relates to an immunoassay and products for the simultaneous detection of HIV-1 antigen analyte and HIV-1 antibody analyte and/or HIV-2 antibody analyte in a test sample.

Acquired immunodeficiency syndrome (AIDS) is a disorder of the immune system associated with opportunistic infections and/or neoplasms which has reached epidemic proportions in the United States as well as in Europe and in central Africa. The epidemiological data suggest that AIDS is caused by at least two types of human immunodeficiency viruses, collectively designated as HIV. HIV type 1 (HIV-1) has been isolated from patients with AIDS and AIDS-related complex (ARC), and from healthy persons at high risk for AIDS. See, for example, F. Barre-Sinoussi et al., Science 220:868-871 91983); M. Popovic et al., Science 224:497-500 (1984); and R. C. Gallo et al., Science 224:500-503 (1984). HIV-1 is transmitted by sexual contact, exposure to blood and certain blood products, or from an infected mother to her fetus or child. P. Piot et al., Science 239:573-579 (1988). The prevalence of HIV-1 antibodies in AIDS and ARC patients and persons at risk is high, and the virus can be isolated from nearly 90% of all seropositive individuals. See, for example, M. G. Samgadharan et al., Science 224:506-508 (1984); and D. Gallo et al., J. Clin. Micro. 25:1291-1294 (1987).

In 1986, a second human immunodeficiency virus, HIV-2, was isolated from patients with AIDS in west Africa. F. Clavel et al., Science 233:343-346 (1986). HIV-2 infections also have been identified in individuals from several

countries outside of west Africa. See, for example, A. G. Saimot et al., Lancet i:688 (1987); M.A. Rey et al., Lancet i:388-389 (1986); A. Werner et al., Lancet i:868-869 (1987); G. Brucker et al., AIDS 2:141 (1988); and K. Marquart et al., AIDS 2:141 (1988). Although at the present time HIV-2 appears to be endemic only in west Africa, it appears likely that, based on the experience with HIV-1, HIV-2 will spread to other parts of the world.

HIV-2 virus is similar to HIV-1 virus in its morphology, cell tropism, interaction with the CD4 cellular receptor, in vitro cytopathic effect on CD4 cells, overall genomic structure and its ability to cause AIDS. F. Clavel, AIDS 1:135-140 (1987). However, HIV-2 differs from HIV-1 in several respects. See F. Clavel, Ibid and R.A. Weiss et al., AIDS 2:95-100 (1988).

Serological tests indicate that HIV-1 and HIV-2 share multiple common epitopes in their core antigens, although their envelope glycoproteins are much less cross-reactive. F. Clavel, supra. This limited cross-reactivity of the envelope antigens may explain the failure of most currently-available serological assays for HIV-1 to react with certain sera from individuals with antibody to HIV-2. F. Denis et al., J. Clin. Micro. 26:1000-1004 (1988). A commercially available assay for HIV-1/HIV-2 antibody, recently available from Abbott Laboratories (Abbott Park, IL 60064), designated as the Abbott HIVAB® HIV-1/HIV-2 (rDNA) EIA, uses recombinant antigens corresponding to the two viral proteins, HIV-1 envelope and HIV-2 envelope. The use of these recombinant antigens allows for the improved detection of anti-HIV-1 and/or anti-HIV-2 containing test samples, while minimizing non-specific reactions largely due to cross reactions with whole virus or viral lysate. The use of at least one recombinant HIV protein to detect HIV antibody in a test sample with the use of labelled recombinant HIV antigens is described in the parent patent applications previously incorporated herein by reference.

Based on the nucleotide analysis of the viral genome the HIV genomic RNA encodes (beginning at the 5' end):

(i) a *gag* gene extending between nucleotides 310 to 1869 and encoding for the internal structure core or nucleocapsid proteins including p24, the most antigenic core protein;

(ii) a *pol* gene extending between nucleotides 1,629 to 4,673 and encoding for the enzyme, reverse transcriptase; and

(iii) an *env* gene extending between nucleotides 5,781 to 8,369 and encoding for the envelope glycoprotein including gp41, the most antigenic envelope protein. Ratner et al., Nature 313:277-284 (1985).

One of the challenges faced by today's medical community is the protection of blood products from contamination by HIV, which has been found in blood products (as well as other human body fluids), and which reportedly has been transmitted in the blood supply. Several assays are available to date, including the assay described in U.S. Patent No. 4,520,113 to Gallo et al.

Also, other assays which can detect HIV antigen or HIV antibody are known. Such assays include the anti-HIV-1/HIV-2 assay described hereinabove and disclosed in the previously referenced parent application, as well as those taught by U.S. Patent No. 4,748,110 to D. Paul, U.S. Patent No. 4,983,529 to J. Stewart et al., and U.S. Patent No. 5,173,399. However, all known federally-approved assays for detection of HIV antigen analyte or HIV antibody analyte are only capable of separately detecting either HIV antigen analyte or HIV antibody analyte in a test sample. No known commercially available, federally-approved assay is available for detection of both HIV antigen analyte and/or HIV antibody analyte in a single assay using a test sample.

The detection of more than one analyte in a test sample usually involves the separate detection of each analyte in a separate assay. Such detection methods have been preferred since they allow for stringent quality assurance determinations to be performed for each analyte to be tested.

Advances in medicine have brought a recognition of new markers for many diseases and clinical conditions, along with the demand for clinical tests for these markers. Laboratories are faced with the problem of providing increasing amounts of tests in a timely manner while attempting to keep costs down. For example, the testing requirements of blood banks have increased dramatically due to the addition of Human T-Leukemia Virus Type 1 (HTLV-1), HIV and Hepatitis C Virus (HCV) to the panel of agents tested in these laboratories on donor blood for the presence of or exposure to these agents.

One possible solution to reducing the laboratory workload brought about as a result of testing requirements, especially in blood banks, is to find ways to combine assays. However, combining assays without compromising their individual performance standards is difficult and more importantly, the problems involved in manufacturing and quality control can be insurmountable.

Several investigators have developed or have attempted to develop assays to simultaneously detect more than one analyte in a test sample. Such an assay would be advantageous since the time involved in detecting more than one analyte in the test sample would diminish considerably, and the cost of each assay would

be lowered since less technical time, reagents, and equipment would be required to perform such an assay.

For example, U.S. Patent No. 4,315,907 to Fridlender et al. teaches a heterogeneous specific binding assay system wherein separation of bound-species from a free-species form of the labeled reagent occurs.

U.S. Patent No. 4,378,344 and EP 027008 to Zahradnik et al. teach a solid phase device for determining the presence of each analyte comprising a receptacle and an insert wherein the presence of each analyte is determined by the claimed assay method.

Great Britain Patent No. 2188418 teaches an assay tray assembly having reaction wells each with openings in the top surface from which a projection is extended and wherein the inner surface of each well sidewall and the outer surface of each projection may be incubated simultaneously for detecting two or more specific substances present in a specimen which has been introduced into the reaction wells.

EPA No. 0 351 248 to applicant IDEXX Corporation discloses a simultaneous immunoassay for feline viruses or HIV in which an antigen and/or antibody member of a single binding pair are detectable. Also, U. S. Patent No. 5,039,604 to Papsidero teaches an immunoassay which simultaneously detects two HTLV or HIV antibodies by adding two different antigens and then a single labelled antibody which is reactive with both antigens. In addition, U.S. Patent No. 4,870,003 to Kortright et al. discloses a solid phase immunoassay for detection of an antigen and/or antibody of a single binding pair utilizing an antigen "spike" of inactivated antigen.

Also, the detection of one or more analytes using two or more solid phases is the subject matter of co-pending U. S. Patent Application Serial No. 574,821 (EP-A-473 065, published March 4, 1992).

The critical factors which have been identified to the development of simultaneous assays are that the two assays to be performed simultaneously must have the same sample volumes, identical incubation times and identical cut-off calculations. Such a simultaneous assay also should be capable of being separately quality controlled for each analyte, both at the manufacturer and at the laboratory using the assay, to ensure the sensitivity, specificity and reproducibility of the immunoassay.

It therefore would be advantageous to provide an assay wherein the presence of more than one HIV analyte could be simultaneously detected, yet each separate analyte to be detected could be individually quality controlled. Such an

assay would be an improvement over other known assays since the simultaneous determinations of the presence of either HIV antigen analyte and/or HIV antibody analyte would be performed in one well, separation of solid phase components would not be required if more than one solid phase was utilized, and the assay could be quality controlled for individual analytes which were to be detected in the simultaneous assay.

SUMMARY OF THE INVENTION

This invention provides an assay to simultaneously detect the presence, if any, of an HIV antigen analyte and/or an HIV antibody analyte which may be present in a test sample. The assay comprises simultaneously contacting the test sample with (i) at least one HIV antigen analyte capture reagent specific for said HIV antigen analyte attached to a solid phase, and (ii) at least one HIV antibody analyte capture reagent specific for said HIV antibody analyte attached to a solid phase, to form a first mixture; incubating said first mixture for a time and under conditions sufficient to form HIV antigen capture reagent/HIV antigen analyte complexes and/or HIV antibody capture reagent/HIV antibody analyte complexes; contacting said complexes with (i) an HIV antigen analyte indicator reagent which comprises a member of a binding pair specific for said HIV antigen analyte labelled with a signal generating compound, and (ii) an HIV antibody analyte indicator reagent which comprises a member of a binding pair specific for the HIV antibody analyte labelled with a signal generating compound, to form a second mixture; incubating said second mixture for a time and under conditions sufficient to form HIV antigen capture reagent/HIV antigen analyte/indicator reagent complexes and/or HIV antibody capture reagent/HIV antibody analyte/indicator reagent complexes; and determining the presence, if any, of said HIV antigen analyte and/or said HIV antibody analyte in the test sample by detecting a signal generated an indication of the presence of either or both analytes in the test sample.

The present invention also provides an assay to simultaneously detect the presence, if any, of an HIV antigen analyte and/or an HIV antibody analyte in a test sample, comprising simultaneously contacting the test sample with: (i) at least one HIV antigen capture reagent specific for said HIV antigen analyte attached to a solid phase, (ii) at least one HIV antibody capture reagent specific for said HIV antibody analyte attached to a solid phase, and (iii) an HIV antigen

analyte indicator reagent which comprises a member of a binding pair specific for said HIV antigen analyte labelled with a signal generating compound, to form a first mixture; incubating said first mixture for a time and under conditions sufficient to form HIV antigen capture reagent/HIV antigen analyte/HIV antigen indicator reagent complexes and HIV antibody capture reagent/HIV antibody analyte complexes; contacting said complexes with an HIV antibody analyte indicator reagent which comprises a member of a binding pair specific for said HIV antibody analyte labelled with a signal generating compound, to form a second mixture; incubating said second mixture for a time and under conditions sufficient to form antibody HIV antibody capture reagent/HIV antibody analyte/HIV antibody analyte indicator reagent complexes; and determining the presence, if any, of said HIV antigen analyte and/or said HIV antibody analyte in the test sample by detecting a signal generated.

The HIV antibody capture reagent and said antigen capture reagent can be attached (bound) to the same or different solid phase. Preferred solid phases include magnetic beads, non-magnetic beads, wells of a reaction tray, microparticles, nylon strips and nitrocellulose strips. The HIV antigen analyte is HIV p24 *gag* antigen and HIV antigen analyte capture reagent is anti-HIV p24 antibody, either monoclonal or polyclonal or recombinantly derived, or fragments thereof. Preferably, the HIV antigen capture reagent is a mixture of monoclonal antibodies secreted by hybridoma cell lines ATCC Deposit Nos. HB 9725 and HB 9726, while the HIV antibody capture reagent is HIV-1 p41 protein , or a mixture of HIV-1 p41 protein and HIV-2 p41 *env* protein. Most preferably, these antigens are recombinantly produced. The signal generating compound of the HIV antigen analyte indicator reagent and the signal generating compound of the HIV antibody analyte indicator reagent is selected from the group consisting of enzymes, luminescent compounds, chemiluminescent compounds and radioactive elements. Additionally, either or both indicator reagents may further comprise a hapten.

The above-described assays can be adapted for the detection of both HIV-1 antigen analyte and HIV-1 and HIV-2 antibody analytes separately. In this assay, the steps comprise (a) contacting the test sample with a solid phase to which at least one anti-HIV p24 antibody and at least one recombinant HIV-1 p41 antigen and at least one HIV-2 p41 antigen have been attached, to form a mixture; (b) incubating said mixture for a time and under conditions sufficient to form anti-HIV p24 antibody/HIV-1 p24 antigen complexes and/or HIV-1 p41 antigen/HIV-1 antibody and/or HIV-2 p41 antigen/anti-HIV-2 p41 antigen complexes; (c)

detecting the presence of HIV p24 antigen in the test sample by: (i) contacting said anti-HIV p24 antibody/HIV p24 antigen complexes with an anti-HIV p24 antibody capable of specifically binding HIV p24 antigens for a time and under conditions sufficient to form anti-HIV p24 antibody/HIV p24 *gag* antigen/anti-HIV p24 antibody complexes, (ii) contacting said anti-HIV p24 antibody/HIV p24 antigen/anti-HIV p24 antibody complexes with an indicator reagent comprising an anti-species antibody labeled with a signal generating compound, and (iii) detecting the signal generated as an indication of the presence of HIV p24 antigen in the test sample; and (d) detecting the presence of anti-HIV-1 p41 antibody and/or HIV-2 p41 antibody in the test sample by: (i) contacting said HIV-1 p41 antigen/anti-HIV-1 p41 antibody complexes and/or HIV-2 p41 antigen/anti-HIV-2 antibody complexes with an indicator reagent comprising HIV-1 p41 antigen labeled with a signal generating compound and HIV-2 p41 antigen labeled with a signal generating compound; and (ii) detecting the signal generated as an indication of the presence of anti-HIV-1 p41 antibody and/or HIV-2 p41 antibody in the test sample. This assay also can be performed using two (or more) solid phases.

Test kits for performing the assays of the present invention also are provided.

DETAILED DESCRIPTION OF THE INVENTION

An assay for the detection of HIV antigen analyte and/or HIV antibody analyte in a test sample is provided. The assay preferably is performed as an immunoassay, although the present invention is not limited to immunoreactive assays. Any assay utilizing specific binding members can be performed. A "specific binding member," as used herein, is a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding member, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments;

antibodies and antibody fragments, both monoclonal and polyclonal; and complexes thereof, including those formed by recombinant DNA methods.

"Analyte," as used herein, is the substance to be detected which may be present in the test sample. The analyte can be any substance for which there exists a naturally occurring specific binding member (such as, an antibody), or for which a specific binding member can be prepared. Thus, an analyte is a substance that can bind to one or more specific binding members in an assay. "Analyte" also includes any antigenic substances, haptens, antibodies, and combinations thereof. As a member of a specific binding pair, the analyte can be detected by means of naturally occurring specific binding partners (pairs) such as the use of intrinsic factor protein in the capture and/or indicator reagents for the determination of vitamin B_{12}, or the use of a lectin in the capture and/or indicator reagents for the determination of a carbohydrate. The analyte can include a protein, a peptide, an amino acid, a hormone, a steroid, a vitamin, a drug including those administered for therapeutic purposes as well as those administered for illicit purposes, a bacterium, a virus, and metabolites of or antibodies to any of the above substances.

The test sample can be a mammalian biological fluid such as whole blood or whole blood components including red blood cells, white blood cells including lymphocyte or lymphocyte subset preparations, platelets, serum and plasma; ascites; saliva; stools; cerebrospinal fluid; urine; sputum; tracheal aspirates and other constituents of the body which may contain or be suspected of containing the analyte(s) of interest. The test sample also can be a culture fluid supernatant, or a suspension of cultured cells. Mammals whose body fluids can be assayed for HIV antigen analyte or HIV antibody analyte according to the present invention include humans and primates, as well as other mammals who are suspected of containing these analytes of interest. It also is contemplated that non-biological fluid samples can be utilized.

The indicator reagent comprises a label conjugated to a specific binding member of each analyte. Each indicator reagent produces a detectable signal at a level relative to the amount of the analyte in the test sample. In a preferred embodiment, each indicator reagent, while comprising a specific binding member of a different analyte, is conjugated to the same signal generating compound (label), which is capable of generating a detectable signal. In general, the indicator reagent is detected or measured after it is captured on the solid phase material. In the present invention, the total signal generated by the indicator reagent(s) indicates the presence of one or more of the analytes in the test

sample. It is contemplated that different signal generating compounds can be utilized in the practice of the present invention. Thus, for example, different fluorescent compounds could be utilized as the signal generating compounds, one for each indicator reagent, and detection could be determined by reading at different wavelengths. Or, a short-lived chemiluminescent compound such as an acridinium or phenanthridinium compound and a long-lived chemiluminescent compound such as a dioxetane can be utilized to generate signals at different times for different analytes. Methods which detail the use of two or more chemiluminescent compounds which are capable of generating signals at different times are the subject matter of co-pending patent application PCT Application No. 92902803.3, filing date December 23, 1991. Acridinium and phenanthridinium compounds are described in co-pending U. S. patent application Serial No. 07/271,763 filed June 23, 1989, which enjoys common ownership and is incorporated herein by reference.

In addition to being either an antigen or an antibody member of a specific binding pair, the specific binding member of the indicator reagent can be a member of any specific binding pair, including either biotin or avidin, a carbohydrate or a lectin, a complementary nucleotide sequence, an effector or a receptor molecule, an enzyme cofactor or an enzyme, an enzyme inhibitor or an enzyme, and the like. An immunoreactive specific binding member can be an antibody, an antigen, or an antibody/antigen complex that is capable of binding either to the analyte as in a sandwich assay, to the capture reagent as in a competitive assay, or to the ancillary specific binding member as in an indirect assay. If an antibody is used, it can be a monoclonal antibody, a polyclonal antibody, an antibody fragment, a recombinant antibody, a mixture thereof, or a mixture of an antibody and other specific binding members. The details of the preparation of such antibodies and their suitability for use as specific binding members are well known to those in the art.

The signal generating compound (label) of the indicator reagent is capable of generating a measurable signal detectable by external means. The various signal generating compounds (labels) contemplated include chromagens; catalysts such as enzymes for example, horseradish peroxidase, alkaline phospatase, and B-galactosidase; luminescent compounds such as fluorescein and rhodamine; chemiluminescent compounds such as acridinium compounds, phenanthridinium compounds and dioxetane compounds; radioactive elements; and direct visual labels. The selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional

substances. A variety of different indicator reagents can be formed by varying either the label or the specific binding member.

The capture reagents of the present invention comprise a specific binding member for each of the analytes of interest which are attached to at least one solid phase and which are unlabeled. Although the capture reagent is specific for the analyte as in a sandwich assay, it can be specific for indicator reagent or analyte in a competitive assay, or for an ancillary specific binding member, which itself is specific for the analyte, as in an indirect assay. The capture reagent can be directly or indirectly bound to a solid phase material before the performance of the assay or during the performance of the assay, thereby enabling the separation of immobilized complexes from the test sample. This attachment can be achieved, for example, by coating the specific binding member onto the solid phases by absorption or covalent coupling. Coating methods, and other known means of attachment, are known to those in the art.

The specific binding member of the capture reagent can be any molecule capable of specifically binding with another molecule. The specific binding member of the capture reagent can be an immunoreactive compound such as an antibody, antigen, or antibody/antigen complex. If an antibody is used, it can be a monoclonal antibody, a polyclonal antibody, an antibody fragment, a recombinant antibody, a mixture thereof, or a mixture of an antibody and other specific binding members.

The "solid phase" is not critical and can be selected by one skilled in the art. Thus, latex particles, microparticles, magnetic or non-magnetic beads, membranes, plastic tubes, walls of wells of reaction trays, glass or silicon chips and tanned sheep red blood cells are all suitable examples. Suitable methods for immobilizing capture reagents on solid phases include ionic, hydrophobic, covalent interactions and the like. In one example of the present invention, 60-well polystyrene reaction trays and 1/4 inch polystyrene beads are utilized, while in another example, a 96-well reaction tray is the only solid phase utilized. It is contemplated that all solid phases be present during the quantitation of signal, thus eliminating the need to separate solid phases for detection of signal.

A "solid phase", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can

include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon (attached to) the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the performance of the assay or during the performance of the assay. The solid phase thus can be a plastic, derivatized plastic, magnetic or non-magnetic metal, glass or silicon surface of a test tube, microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

It is contemplated and within the scope of the invention that the solid phase also can comprise any suitable porous material with sufficient porosity to allow access by detection antibodies and a suitable surface affinity to bind antigens. Microporous structures are generally preferred, but materials with gel structure in the hydrated state may be used as well. Such useful solid supports include:

Natural polymeric carbohydrates and their synthetically modified, cross-linked or substituted derivatives, such as agar, agarose, cross-linked alginic acid, substituted and cross-linked guar gums, cellulose esters, especially with nitric acid and carboxylic acids, mixed cellulose esters, and cellulose ethers; natural polymers containing nitrogen, such as proteins and derivatives, including cross-linked or modified gelatins; natural hydrocarbon polymers, such as latex and rubber; synthetic polymers which may be prepared with suitably porous structures, such as vinyl polymers, including polyethylene, polypropylene, polystyrene, polyvinylchloride, polyvinylacetate and its partially hydrolyzed derivatives, polyacrylamides, polymethacrylates, copolymers and terpolymers of the above polycondensates, such as polyesters, polyamides, and other polymers, such as polyurethanes or polyepoxides; porous inorganic materials such as sulfates or carbonates of alkaline earth metals and magnesium, including barium sulfate, calcium sulfate, calcium carbonate, silicates of alkali and alkaline earth metals, aluminum and magnesium; and aluminum or silicon oxides or hydrates, such as clays, alumina, talc, kaolin, zeolite, silica gel, or glass (these materials may be used as filters with the above polymeric materials); and mixtures or copolymers of the above classes, such as graft copolymers obtained by initializing polymerization of synthetic polymers on a pre-existing natural polymer. All of these materials may be used in suitable shapes, such as films,

sheets, or plates, or they may be coated onto or bonded or laminated to appropriate inert carriers, such as paper, glass, plastic films, or fabrics.

The porous structure of nitrocellulose has excellent absorption and adsorption qualities for a wide variety of reagents including monoclonal antibodies. Nylon also possesses similar characteristics and also is suitable.

It is contemplated that such porous solid supports described hereinabove are preferably in the form of sheets of thickness from about 0.01 to 0.5 mm, preferably about 0.1 mm. The pore size may vary within wide limits, and is preferably from about 0.025 to 15 microns, especially from about 0.15 to 15 microns. The surfaces of such supports may be activated by chemical processes which cause covalent linkage of the antigen or antibody to the support. The irreversible binding of the antigen or antibody is obtained, however, in general, by adsorption on the porous material by poorly understood hydrophobic forces.

Preferred solid phase materials for flow-through assay devices include filter paper such as a porous fiberglass material or other fiber matrix materials. The thickness of such material is not critical and will be a matter of choice, largely based upon the properties of the sample or analyte being assayed, such as the fluidity of the test sample.

To change or enhance the intrinsic charge of the solid phase, a charged substance can be coated directly to the material or onto microparticles which then are retained by a solid phase support material. Alternatively, microparticles can serve as the solid phase, by being retained in a column or being suspended in the mixture of soluble reagents and test sample, or the particles themselves can be retained and immobilized by a solid phase support material. By "retained and immobilized" is meant that the particles on or in the support material are not capable of substantial movement to positions elsewhere within the support material. The particles can be selected by one skilled in the art from any suitable type of particulate material and include those composed of polystyrene, polymethylacrylate, polypropylene, latex, polytetrafluoroethylene, polyacrylonitrile, polycarbonate, or similar materials. The size of the particles is not critical, although it is preferred that the average diameter of the particles be smaller than the average pore size of the support material being used. Thus, embodiments which utilize various other solid phases also are contemplated and are within the scope of this invention. For example, ion capture procedures for immobilizing an immobilizable reaction complex with a negatively charged polymer, described in co-pending U. S. Patent Application Serial No. 150,278 (corresponding to EP Publication No. 0326100), and U. S. Patent Application

Serial No. 375,029 (EP Publication No. 0406473), which enjoy common ownership and are incorporated herein by reference, can be employed according to the present invention to effect a fast solution-phase immunochemical reaction. An immobilizable immune complex is separated from the rest of the reaction mixture by ionic interactions between the negatively charged poly-anion/immune complex and the previously treated, positively charged porous matrix and detected by using various signal generating systems previously described, including those described in chemiluminescent signal measurements as described in co-pending U.S. Patent Application Serial No. 921,979 corresponding to EPO Publication No. 0 273,115, which enjoys common ownership and which is incorporated herein by reference.

Also, the methods of the present invention can be adapted for use in systems which utilize microparticle technology including automated and semi-automated systems wherein the solid phase comprises a microparticle. Such systems include those described in pending U. S. Patent Application 425,651 and U. S. Patent No. 5,089,424, which correspond to published EPO applications Nos. EP 0 425 633 and EP 0 424 634, respectively, and U.S. Patent No. 5,006,309 all of which enjoy common ownership and are incorporated herein by reference.

In the practice of one embodiment of the present invention, a test sample suspected of containing any of the HIV antigen analyte or HIV antibody analytes of interest is simultaneously contacted with a solid phase to which a first specific binding member of a first analyte is attached, and a solid phase to which a first specific binding member of a second analyte has been attached, thereby forming a mixture. The specific binding members serve as capture reagents to bind the analyte(s) to the solid phases. If the specific binding member is an immunoreactant, it can be an antibody, antigen, or complex thereof, specific for each analyte of interest. If the specific binding member is an antibody, it can be a monoclonal or polyclonal antibody, an antibody fragment, a recombinant antibody, as well as a mixture thereof, or a mixture of an antibody and other specific binding members. This mixture is incubated for a time and under conditions sufficient for a binding reaction to occur and which incubation results in the formation of capture reagent/first analyte complexes of the first analyte if it is present in the test sample, and/or the formation of capture reagent/second analyte complexes of the second analyte if it is present in the test sample.

Then, an indicator reagent for each analyte is contacted with the complexes. The indicator reagent for the first analyte comprises a specific binding member of the first analyte of interest which has been labelled with a

signal generating compound. The indicator reagent for the second analyte comprises a specific binding member of the second analyte of interest which has been labelled with the same signal generating compound as the indicator reagent for the first analyte, thereby forming a second mixture. This second mixture is incubated for a time and under conditions sufficient to form capture reagent/first analyte/indicator reagent complexes and/or capture reagent/second analyte/indicator reagent complexes. The presence of either analyte is determined by detecting the signal generated in connection with the complexes formed on the solid phase as an indication of the presence of one or more analytes in the test sample. If the indicator employs an enzyme as the signal generating compound (label), then the signal can be detected visually or measured spectrophotometrically. Or, the label can be detected by the measurement of fluorescence, chemiluminescence, radioactive energy emission, etc., depending on the label used to generate the signal.

The capture reagents can be attached to the same solid phase, or can be attached to different solid phases. It is contemplated that all capture reagents could be attached to the same solid phase, or that each capture reagent could be attached to a separate solid phase, or that combination of capture reagents could be attached to separate solid phases. For example, if microparticles were the solid phase of choice, then separate microparticles could have at least one capture reagent(s) attached to it. A mixture of microparticles (solid phases) could be used to capture the various analytes which may be present in the test sample by using the mixture of microparticles. It is contemplated that different ratios of capture reagents attached to solid phases could be utilized in such an assay, to optimize analyte(s) detection.

In the embodiment described hereinabove, it is preferred that the specific binding member used as a capture reagent for the HIV-1 antibody analyte be HIV-1 p41 antigen , and that the specific binding member used as the capture reagent for the HIV-1 antigen analyte be anti-HIV-1 p24 antibody. It is most preferred that the HIV-1 p41 used be a recombinantly prepared antigen (protein). Also, it is preferred that the specific binding member for the antibody analyte indicator reagent is HIV-1 p41 antigen, labelled with an enzyme, and that the specific binding member for the antigen analyte indicator reagent is anti-HIV p24 antibody, labelled with an enzyme. It is most preferred this HIV-1 p41 antigen be recombinantly produced, and that the enzyme be horseradish peroxidase (HRPO).

In another embodiment of the present invention, a test sample suspected of containing any of the analytes of interest is simultaneously contacted with a first solid phase to which a first specific binding member of a first analyte and a first specific binding member of a second analyte have been attached, an indicator reagent for the first analyte comprising a specific binding member for the first analyte labelled with a signal generating compound and an indicator reagent for the second analyte comprising a specific binding member for the second analyte labelled with a signal generating compound, to form a mixture. The specific binding members serve as capture reagents to bind the analyte(s) to the solid phases. If the specific binding member is an immunoreactant, it can be an antibody, antigen, or complex thereof, specific for each analyte of interest. If the specific binding member is an antibody, it can be a monoclonal or polyclonal antibody, an antibody fragment, a recombinant antibody, as well as a mixture thereof, or a mixture of an antibody and other specific binding members. The indicator reagents comprise specific binding members of the first and second analytes of interest which have been labelled with a signal generating compound. This mixture is incubated for a time and under conditions sufficient for a binding reaction to occur and which incubation results in the formation of capture reagent/first analyte/indicator reagent complexes of the first analyte and/or capture reagent/second analyte/indicator reagent complexes of the second analyte, if either or both the first or second analyte are present in the test sample. The presence of either analyte is determined by detecting the signal generated in connection with the complexes formed on either or both solid phases as an indication of the presence of the first analyte and/or the second analyte in the test sample. If the indicator employs an enzyme as the signal generating compound (label), then the signal can be detected visually or measured spectrophotometrically. Or, the label can be detected by the measurement of fluorescence, chemiluminescence, radioactive energy emission, etc., depending on the label used. Also, it is contemplated that the assay can include the use of a hapten-anti-hapten system, in which case the indicator reagent can further comprise a hapten such as biotin. The use of a biotin/anti-biotin system for assays is the subject matter of co-pending U.S. Patent Application Serial No. 687,785 which corresponds to published European Patent Application No. 0160900 (published November 13, 1985), which enjoys common ownership and is incorporated herein by reference.

In the embodiment described hereinabove, it is preferred that the specific binding member used as a capture reagent for the HIV-1 antibody analyte be HIV-

1 p41 antigen, and that the specific binding member used as the capture reagent for the HIV-1 antigen analyte be anti-HIV-1 p24 antibody. It is most preferred that the HIV-1 p41 used be a recombinantly prepared antigen (protein). Also, it is preferred that the specific binding member for the antibody analyte indicator reagent is HIV-1 p41 antigen, labelled with an enzyme. It is most preferred this HIV-1 p41 antigen be recombinantly produced, and that the enzyme be horseradish peroxidase (HRPO). Solid phases preferred include a magnetic or non-magnetic bead, a well of a reaction tray, and microparticles, either alone or in any combination.

Positive and negative controls can be included in the assay of the present invention to ensure reliable results. A blank solid phase(s), to which no capture reagent has been attached, can be utilized as the negative reagent control. Positive controls can include a positive control for each analyte which control is tested separately, and a combined positive control wherein the presence of all analytes to be detected in the assay are determined.

As previously stated, it is preferred that recombinantly-prepared antigens be used in the assay. Although it is known to use inactivated whole virus which has been cultured in a cell line capable of virus replication as a reagent in an assay for HIV antibody, uninfected persons may occasionally give false positive results. The detection of false positive (reactive) results in screening assays of blood products is one of the most pressing problems associated with many known methods of HIV detection. These false problems often are associated with nonspecific binding of immunoglobulins to cellular protein in the viral isolates. These reagents also have the drawback of potentially dangerous manufacturing methods, since culturing of live virus in vitro and isolation and deactivation processes can expose workers to the HIV virus.

Although the most specific test for HIV infection remains isolation of the HIV virus, this is an impractical method for large scale use due to the complexity, difficulty and time spent isolating HIV in culture.

The use of recombinantly-produced HIV protein (antigen) as an antigen reagent in assay methods may solve these problems. Recombinant proteins are non-infectious and therefore the production and isolation of such proteins would be safer than culturing the whole virus. Also, the use of pure viral protein obtained by recombinant methods should eliminate some of the false positive reactions due to nonspecific reactions with contaminating proteins. Further, standardization of reagents should improve the specificity and predictive value of the assay.

Expression of HIV gp41 or parts of HIV gp41 have demonstrated the utility of recombinant DNA (rDNA) derived HIV envelope sequences in diagnostic assays. Wood et al., Cold Spring Harbor Symposium on RNA Tumor Viruses, Cold Spring Harbor, New York, May 22-26 (1985); Chang et al., *Biotechnology* 3:905-909 (1985); Crowl et al., *Cell* 41:979-986 (1985); Cabradilla et al., *Biotechnology* 3:128-133 (1986). While it is general knowledge that viral proteins expressed in E. coli or other organisms have potential utility in diagnostic assays, development of immunoassays using these reagents, which also will have the specificity and sensitivity equal to or greater than the native viral proteins derived from the cell culture has been a difficult task. Further, the expression of HIV *gag* proteins in E. coli have indicated that the HIV *gag* proteins produced by rDNA technology could have potential diagnostic value. Wood et al., Cold Spring Harbor Symposium on RNA Tumor Viruses, Cold Spring Harbor, New York, May 22-26 (1985); Dowbenko et al., *PNAS USA* 82:7748-7752 (1985); Ghrayeb et al., *DNA* 5:93099 (1986); Steimer et al., *Virology* 150:283-290 (1986).

The present invention utilizes recombinantly-produced HIV envelope proteins as assay reagents. The cloning of the HIV genome and expression of HIV envelope and core protein in E. coli, the purification and characterization of gp41 and p24, and various assay formats which utilize these recombinant proteins are described in EP-A-386 713, published September 12, 1990 (U.S. Patent Application Serial No. 07/020,282 filed February 27, 1987 and previously incorporated herein by reference, from which this present invention claims priority). Briefly, HIV-infected HT-9 cells were harvested and total cellular DNA was isolated an subjected to digestion. The DNA segments encoding for the core protein and for the envelope glycoprotein were further subcloned into bacterial expression vectors using well-known recombinant technology. The priority application also teaches that, in the detection of HIV-1 antibody, the use of recombinant antigens as the capture reagent and the indicator reagent allows for the detection of anti-HIV-1 antibodies of different immunoglobulin classes. These immunoglobulin classes include IgG, IgA, IgE and IgM. The detection of anti-HIV-1 IgG, IgM and IgA using the Abbott HIVAB® HIV-1/HIV-2 (rDNA) EIA assay has been described in an abstract by J. L. Gallarda et al., 5th Annual Forum on AIDS, Hepatitis and Other Blood-Borne Diseases, Atlanta, Georgia, March 29-April 1, 1992.

It is contemplated and within the scope of the present invention that recombinant antigens produced in heterologous sources can be utilized in the

assay and will contribute an even greater lessening of false positive results. For example, if an E. coli prepared recombinant antigen such as p41 is used as the capture reagent, then a recombinant antigen p41 produced in any suitable source different than E. coli, such as in a suitable yeast host or other suitable host such as B. megaterium, can be used.

Further, although the present invention preferably utilizes recombinantly produced antigens, it is well within the scope of the invention to utilize synthetic proteins instead of recombinantly produced antigens. Thus, various synthetically prepared HIV peptides, of varying length, can be used.

The present invention also utilizes antibodies which specifically bind to HIV antigen analytes. In a preferred embodiment, anti-HIV p24 antibody is used. In a most preferred embodiment, a mixture of monoclonal antibodies, both specific for HIV p24 antigen, is used. In this mixture, one monoclonal antibody which specifically binds to an epitope on HIV-1 p24 to which epitope human anti-HIV-1 p24 IgG does not competitively bind is used with another monoclonal antibody which specifically binds to a different epitope of HIV-1 p24 to which different epitope human anti-HIV-1 p24 IgG does competitively bind. Further, the monoclonal antibody which does not competitively bind human anti-HIV-1 p24 IgG also specifically binds to HIV-2 p24 antigen. These monoclonal antibodies and their use in HIV antigen assays are the subject matter of co-pending U. S. Patent No. 5,173,399, issued December 22, 1992. These monoclonal antibodies are designated as 31-42-19 and 31-90-25. Hybridoma cell line 31-42-19 producing monoclonal antibody 31-42-19 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9726. Hybridoma cell line 31-90-25 producing monoclonal antibody 31-90-25 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9725. The use of these monoclonal antibodies as antibody fragments in HIV antigen assays also has been described in U.S. Patent No. 5,173,399.

It is contemplated and within the scope of the invention that the detection of HIV-2 antigen is possible with the assay of the invention. In this assay format, HIV-2 p41 would be attached to a solid support as the HIV-2 antigen capture reagent, in addition to the previously-described HIV-1 p41 antigen capture reagent and HIV-1 antibody capture reagents. The solid support can be the same solid support to which all other capture reagents are attached, it can be

the same solid support to which HIV-1 recombinant antigens have been attached, or it can be attached to a solid phase to which no other capture reagent (except for HIV-2 p41) has been attached. The assay procedure would be the same as described hereinabove for the various embodiments of the invention. The HIV-2 antibody analyte indicator reagent would comprise HIV-2 p41 antigen attached to a detectable label. In a preferred embodiment, it is preferred that recombinantly prepared HIV-2 p41 is utilized. The sequence for the HIV-2 virus (including p41 antigen) is described in EP-A 347,365, published December 20, 1989 to Diagen Corporation, which is incorporated herein by reference. A most preferred HIV-2 recombinant antigen encodes the first 104 amino acids of the HIV-2 p41 antigen. The resulting plasmid designated as pJC104 expresses the HIV-2 *env* protein as a fusion with CKS protein. This plasmid encodes a recombinant protein containing the first 239 amino acids of the CKS protein, 13 amino acids from the pTB210N multiple restriction site linker, 104 amino acids from the HIV-2 *env* protein (amino acids 506-609), and an additional 15 amino acids from the pTB210N multiple restriction site linker, following the methods disclosed by Bolling and Mandecki, "CKS Method of Protein Synthesis", EP-A-475 182 published March 18, 1992 (U.S. Patent Application Serial No. 167,067, filed March 11, 1988, which enjoys common ownership and is incorporated herein by reference).

EXAMPLES

Example 1

Coating Procedure Using Two Solid Phases

This procedure utilized 1/4 inch polystyrene beads (available from Abbott Laboratories, Abbott Park, IL 60064) and a 60-well polystyrene reaction tray (available from Abbott Laboratories, Abbott Park, IL 60064). Two different anti-HIV-1 p24 monoclonal antibodies were coated on the beads, as follows. The beads were coated at a concentration of 8 µg/ml (approximately 1.6 µg/ml/bead) in a 0.25 M sodium citrate buffer (pH 7.2) for two hours at 45°C. The beads then were washed in the 0.25 M sodium citrate buffer (pH 7.2), and then they were reacted with a detergent solution containing 0.1% Triton X-100® (polyoxyethylene ether, available from Sigma Chemical Co., St. Louis, MO) for one hour at 45°C. The beads next were blocked with 1% bovine serum

albumin (BSA) in 0.25 M sodium citrate buffer (pH 7.2) for 30 minutes at 45°C, and then overcoated with 2% sucrose, 1% phosphate glass for 15 minutes at 15-30°C in 0.25 M sodium citrate buffer and allowed to dry. The two monoclonal antibodies used are designated as 31-42-19 and 31-90-25. They are the subject matter of a patent application U.S. Patent Application Serial No. 07/204,798 that describes their development and uses, previously incorporated herein by reference. Their use also has been described in U.S. Patent No. 7,204,798, which enjoys common ownership and is incorporated herein by reference. Hybridoma cell line 31-42-19 producing monoclonal antibody 31-42-19 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9726. Hybridoma cell line 31-90-25 producing monoclonal antibody 31-90-25 was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 26, 1988 and has been accorded ATCC Deposit No. HB 9725.

Next, the wells of the 60-well reaction tray were coated with HIV antigen, as follows. The recombinant protein HIV-1 p41 *env* protein designated as pTB319 was added to each well at a concentration of 1 µg/ml in 0.1 M 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS buffer, pH 11), and incubated for two hours at 40°C. The wells then were washed twice with 400 µl of phosphate buffered saline (PBS, pH 7.5), reacted with 0.1% Tween-20® for one hour at 40°C, and then blocked with 3% BSA in PBS for one hour at 40°C. The wells next were overcoated with 5% sucrose in PBS for 20 minutes at room temperature and allowed to dry.

The pTB319 plasmid producing recombinant protein pTB319 is the subject matter of a patent application to Bolling and Mandecki, "CKS Method of Protein Synthesis," U.S. Patent Application Serial No. 167,067, filed March 11, 1988, previously incorporated herein by reference. pTB319 was produced by inserting a synthetically-produced DNA fragment which encoded the carboxy terminal 42 amino acids of HIV-1 p120 into the plasmid pTB315, as described in Bolling and Mandecki, ibid.

Example 2

Simultaneous Assay for HIV antigen and HIV antibody

The two solid phases prepared as described in Example 1 were used in an assay for detection of HIV antigen and/or HIV antibody in a test sample, as

follows. An HIV-1 seroconversion panel, which contained 65 serum samples derived from nine HIV-1 infected individuals undergoing seroconversion, was used in the assay. Each serum sample was diluted in a separate well of the 60-well tray previously prepared in Example 1 by adding 150 µl of the serum sample to 50 µl of specimen diluent, which contained 2% Tween 20® (polyoxyethylenesorbitan, available from Sigma Chemical Co., St. Louis, MO). Then, a bead previously coated with anti-HIV p24 antibodies as described in Example 1 was placed in each well containing a serum sample. The wells of each tray were incubated for 60 minutes at 40°C under continuous rotation. Following incubation, each well of the 60-well reaction tray was washed with 15 ml of deionized water (dH_2O) in the Abbott Parallel Processing Center™ (PPC, available from Abbott Laboratories, Abbott Park, IL). 200 µl of an HIV p24 antibody probe reagent (rabbit polyclonal $F[ab']_2$ anti-HIV-1 [active ingredient: anti-p24 antibody at a concentration of 2 to 6 µg/ml] in an antibody diluent (2.25% BSA, 7.5% calf serum, 7.5% goat serum, 25% human recalcified plasma, 0.1% sodium azide) was added to each well/bead and then the resulting mixture was incubated for 60 minutes at 40°C without rotation. Each bead/well in the reaction tray was washed with 15 ml of dH_2O. Then, 200 µl of conjugate diluent (0.18% Tris, 1.19 % Tris-HCl, 0.38% NaCl, 9.0% calf serum, 0.9% goat serum, 10.0% human calcified plasma, 4.5% Triton X-100®, 0.013% gentamicin sulfate, 0.009% thimerosal) which contained a mixture of recombinant

HIV-1 p41 antigen labelled with horseradish peroxidase (pTB319 coupled to HRPO), and HRPO-labelled goat anti-rabbit IgG antibody were added to each bead/well of the reaction tray and allowed to incubate for 60 minutes at 40°C without rotation. Each bead/well of the 60-well reaction tray was washed with 15 ml of dH_2O as previously described. Then, 300 µl of o-phenylenediamine-2HCl (OPD) was added to each well/bead and then was incubated for 30 minutes at room temperature in the dark. The reaction then was stopped by adding 300 µl of a stopping reagent (1 N H_2SO_4) to each well/bead. The reaction was read using the Abbott PPC which measured the optical density of the reaction at 492 nm using a 630 nm reference. The cutoff value was established as 0.1 OD + mean OD of the negative control. Thus, serum samples were considered reactive (positive) if the sample to cutoff value was greater than 1.

All 65 serum samples from the 9 individuals described hereinabove were tested following this procedure. The results obtained then were compared to the results obtained for the same serum sample when using an HIV antigen assay

(HIVAG®, available from Abbott Laboratories, Abbott Park, IL) and an HIV antibody assay (Human Immunodeficiency Virus Types 1 and 2: E. coli and B. megaterium, recombinant antigen, Abbott HIVAB® HIV-1/HIV-2 (rDNA) EIA; available from Abbott Laboratories, Abbott Park, IL) following manufacturer's directions as provided in each product insert. The data are reported in Table 1, wherein "OD" refers to the optical density reading, "S/CO" means sample/cut-off value, "Result." refers to the interpretation of the test, "HIV-1/2 Ab HIV-1 Ag Comb" designates the assay of the invention, "HIV-1/2 Ab" designates the HIVAB® HIV-1/HIV-2 (rDNA) EIA assay and "HIV-1 Ag" designates the Abbott HIVAG® assay.

TABLE 1

Sample ID		HIV-1/2 Ab HIV-1 Ag Combination		HIV-1/2 Ab		HIV-1 Ag	
		S/CO	Result	S/CO	Result	S/CO	Result
SV0021	1	1.34	+	0.33	-	1.10	+
	2	1.92	+	2.55	+	1.80	+
	3	3.15	+	6.32	+	2.20	+
	4	1.79	+	3.65	+	0.60	-
SV0031	5	0.83	-	0.13	-	0.40	-
	6	0.87	-	0.13	-	0.30	-
	7	0.86	-	0.13	-	0.35	-
	8	0.78	-	0.15	-	0.35	-
	9	0.95	-	0.17	-	0.40	-
	10	0.80	-	0.13	-	0.38	-
	11	0.62	-	0.10	-	0.35	-
	12	3.40	+	9.08	+	13.20	+
	13	3.40	+	10.09	+	8.50	+
	14	3.40	+	3.00	+	10.50	+
	15	3.40	+	3.28	+	6.40	+
	16	1.87	+	5.25	+	3.20	+
	17	1.52	+	7.34	+	0.80	-
	18	1.38	+	7.28	+	1.20	+
SV0051	19	0.97	-	0.40	-	1.70	+
	20	2.21	+	1.49	+	15.00	+
	21	2.55	+	2.66	+	7.90	+
	22	2.54	+	7.30	+	2.20	+
	23	2.57	+	5.51	+	1.80	+
	24	1.89	+	3.81	+	0.40	-
SV0091	25	2.40	+	0.24	-	18.80	+
	26	3.40	+	0.44	-	71.60	+
	27	3.40	+	2.81	+	5.20	+
	28	3.40	+	2.75	+	1.90	+
	29	2.71	+	2.43	+	0.65	-
	30	2.23	+	2.25	+	0.65	-
	31	1.54	+	3.72	+	0.49	-
	32	1.35	+	10.57	+	0.41	-
	33	nt*					
SV0111	34	3.40	+	0.12	-	32.80	+
	35	3.40	+	0.45	-	42.90	+
	36	3.40	+	11.23	+	20.70	+
	37	2.35	+	4.16	+	1.40	+
	38	1.82	+	3.81	+	1.10	+
	39	1.71	+	3.34	+	0.80	-
	40	1.40	+	5.49	+	0.72	-

Sample ID		HIV-1/2 Ab HIV-1 Ag Combination		IV-1/2 Ab		HIV-1 Ag	
		S/CO	Result	S/CO	Result	S/CO	Result
SV0161	41	1.01	+	0.12	-	1.44	+
	42	1.21	+	1.14	-	3.82	+
	43	2.17	+	0.79	-	8.41	+
	44	3.40	+	10.25	+	11.80	+
	45	2.48	+	2.82	+	0.65	-
	46	1.51	+	2.37	+	0.48	-
SV0061	47	1.13	+	0.20	-	2.90	+
	48	1.23	+	0.26	-	3.60	+
	49	3.33	+	1.93	+	10.90	+
	50	3.09	+	1.72	+	13.50	+
	51	1.93	+	0.96	-	7.10	+
	52	1.88	+	1.39	+	4.40	+
	53	1.30	+	1.60	+	1.90	+
	54	1.45	+	3.10	+	0.97	-
	55	1.22	+	4.28	+	0.98	-
SV0071	56	0.97	-	0.13	-	0.40	-
	57	0.84	-	0.14	-	0.40	-
	58	3.19	+	2.97	+	2.30	+
	59	1.82	+	2.37	+	0.52	-
	60	0.97	-	2.84	+	0.42	-
SV0081	61	0.80	-	0.12	-	0.45	-
	62	0.85	-	0.15	-	0.32	-
	63	0.83	-	0.14	-	0.49	-
	64	1.41	+	3.40	+	0.43	-
	65	1.10	+	4.96	+	0.37	-
	66	1.23	+	8.58	+	0.37	-
No. Positive/ No. Tested		51/65		41/65		35/65	

*nt: This sample of the seroconversion panel was unavailable for testing.

The data from Table 1 indicates that the method of the present invention had greater sensitivity than either the HIV-1/2 Ab or the HIV-1 Ag test when the results from the three individual tests were compared separately to each other. It is expected that the assay can be optimized even further to detect both HIV p24 antigen which is present early in the course of infection and also in the final stages of HIV infections, as well as HIV antibodies which appear later in infection at the time of seroconversion.

Example 3

Coating Procedure For One Solid Phase

In this procedure, only one solid phase was coated with HIV antigen and HIV antibody, as follows. Into each well of a 96-well microtiter plate (Immulon 4®, available from Dynatech, Alexandria VA) monoclonal antibody 31-42-19, monoclonal antibody 31-90-25 and recombinant HIV-1 p41 antigen designated as pTB 319 (as described in Example 1) were coated at a concentration of 1 µg/ml each in 0.1 M carbonate buffer (pH 9.5) for two hours at room temperature. The wells next were blocked with 300 µl of blocking reagent (comprising 5% non-fat dry milk, 10 mM Tris [pH 8.0] 150 mM NaCl and 0.05% Tween-20®) for one hour at room temperature.

Example 4

HIV Antigen/Antibody Assay Using One Solid Phase

The solid phase prepared as described in Example 3 was used in an assay to detect the presence of HIV antigen and/or HIV antibody in a test sample, as follows. Each serum sample of a 12-member seroconversion panel (Panel G available from Boston Biomedica Inc., Boston MA) as well as positive and negative controls were tested. 150 µl of each serum sample or positive or negative control was diluted in a separate well of the microtiter plate with 50 µl of specimen diluent (containing 15 µl of Triton X-100® and 35 µl of blocking reagent, as described in Example 3) and incubated for 60 minutes at room temperature without rotation. After incubation the wells were washed with eight cycles of 300 µl of washing buffer (0.05% non-fat dry milk, 10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween 20®) using a Nunc 8-channel "Immunowash" manifold (available from Nunc, Denmark). Next, 175 µl of an HIV p24 antibody probe reagent (rabbit polyclonal $F[ab']_2$ anti-HIV-1 [active

ingredient: anti-p24 antibody at a concentration of 2 to 6 µg/ml] in an antibody diluent (2.25% BSA, 7.5% calf serum, 7.5% goat serum, 25% human recalcified plasma, 0.1% sodium azide) were added to each well and incubated for 60 minutes at room temperature without rotation. After incubation the wells were washed with eight cycles of wash buffer as previously described. Then, 150 µl of conjugate diluent (as described in Example 2) which contained a mixture of recombinant HIV-1 p41 protein (pTB 319) labelled with HRPO and HRPO-labelled goat anti-rabbit IgG (previously described in Example 2) were added to each well and incubated for 60 minutes at room temperature without rotation. The wells were washed with eight cycles of wash buffer (described previously herein) and then rinsed with dH_2O. Then, 125 µl of OPD substrate was added to each well and the wells were incubated at room temperature for 10 minutes in the dark. The reaction was stopped by adding 125 µl of stopping reagent (previously described in Example 2). The absorbance of each well was read at 490 nm with a 630 nm reference. The cutoff value of 0.025 OD + mean OD of the negative control was established. Samples were considered reactive (positive) when the sample/cutoff value was greater than 1

The data from these assays are presented in Table 2. In Table 2, "OD" refers to the optical density reading, "S/CO" means sample/cut-off value, "Result." refers to the interpretation of the test, "NC" refers to negative control and "PC" refers to the positive control.

TABLE 2

	HIV-1/2 Ab HIV-1 Ag Combination		HIV-1/2 Ab		HIV-1 Ag	
	S/CO	Result	S/CO	Result	pg/ml*	Result
NC	0.85	-				
NC	0.72	-				
NC	0.82	-				
PC	1.35	+				
PC	1.13	+				
1	1.22	+	0.09	-	>200	+
2	1.64	+	0.23	-	>200	+
3	2.33	+	2.55	+	>200	+
4	4.04	+	4.07	+	155	+
5	2.42	+	1.92	+	40	+
6	1.03	+	1.95	+	5	+
7	1.09	+	4.87	+	0	-
8	1.57	+	7.31	+	0	-
9	1.61	+	7.31	+	0	-
10	1.79	+	9.40	+	0	-
11	3.76	+	>17.85	+	0	-
12	5.62	+	>17.85	+	0	-
TOTALS	12/12		10/12		6/12	

*pg/ml - picograms/ml

As the data from Table 2 demonstrate, the assay of the invention was capable of detecting the presence of HIV antibody and/or HIV antigen in the seroconversion panel. Compared individually to the HIV-1/2 Antibody test, and HIV-1 Antigen test, the method of the present invention was more sensitive at detection than either test alone, based on detection of either antigen or antibody. When the results from Table 2 of the method of the present invention are compared to the combined results of the HIV-1/2 antibody test and the HIV antigen test, the method of the present invention was able to detect all specimens that were reactive by either test.

Example 5

Coating Microparticles Simultaneously With HIV Antibody and HIV Antigen Capture Reagents

Both monoclonal anti-HIV p24 antibodies previously described (31-42-19 and 31-90-25) and the recombinant HIV-1 p41 antigens previously described (HIV-1 p41 recombinant protein pTB319, and HIV-2 p41 recombinant protein pJC104) are together and simultaneously coated onto a uniform 0.5% suspension (wt/volume) of polystyrene microparticles (available from Seradyne Inc., Indianapolis, Indiana) at concentrations of 150 µg/ml each in 0.01 M carbonate buffer (pH 9.5) for two hours at room temperature (15-30°C). The suspension of microparticles is briefly centrifuged and the microparticle pellet is resuspended in 0.05M Tris buffer (pH 8.0) to wash away excess uncoupled protein. This washing is repeated until no uncoupled protein remains. After blocking the microparticles with 10 mg/ml casein in 0.01 M Tris (pH 8.0), 0.15 M NaCl at 56°C for 18-24 hours, the microparticles again are washed as described herein and diluted to 0.015% suspension (wt./volume) in 0.05 M Tris (pH 8.0), 0.15 M NaCl, 1% BSA, 15% sucrose and 0.1% sodium azide.

Example 6

Simultaneous Detection of HIV Antibody and HIV Antigen Using Microparticles

The Abbott IM$_X$® Microparticle Enzyme Immunoassay (MEIA) system is used, although any system which employs microparticles can be used. The Abbott IM$_X$® MEIA system is thoroughly described in the Abbott IM$_X$® Operation and Customer Training Manuals (available from Abbott Diagnostic Division, Abbott

Laboratories, Abbott Park, IL). In this assay, 100 µl of the 0.15% suspension prepared in Example 5 is mixed together with 100 µl of test sample suspected of containing HIV-1 and/or HIV-2 antibody and/or HIV-1 antigen, and incubated at 40°C for ten minutes in an Abbott IM$_X$® reaction cell to form a reaction mixture. HIV antibodies and/or HIV antigens bind to the microparticles in an antibody/antigen/microparticle complex. 150 µl of the reaction mixture is transferred onto a glass fiber matrix to which the microparticles are retained in an irreversible binding. The antibody/antigen/microparticle complexes then are reacted with 50µl of a probe consisting of biotinylated recombinant HIV-1 and HIV-2 recombinant p41 antigens (pTB319 and pJC104, previously described) and biotinylated F(Ab')$_2$ anti-HIV-1 p24 in 0.05M Tris 9 pH 8.0), 2% BSA, 0.25% saponin and 0.1% sodium azide at 40°C for ten minutes. 50 µl of an antibody conjugate consisting of goat anti-biotin alkaline phosphatase in 0.1 M Tris (pH 8.0), 0.5M NaCl, 0.9% Brij-35®, 1.0% BSA and 0.1% sodium azide then is allowed to react with the biotin probe/antibody/antigen/microparticle complexes for ten minutes at 40°C. Then, these microparticle complexes are washed six times with 0.05 M Tris (pH 8.0), 0.3 M NaCl and 0.1 % sodium azide, the biotin probe/antibody/antigen microparticle complex is reacted with 50 µl of the substrate methylumbelliferyl phosphase (MUP, Abbott Laboratories, Abbott Park, IL), and the fluorescence of the product , methylumbelliferon, (MU) is measured. The rate of MU production is proportional to the concentration of analyte(s) in the test sample.

Example 7

Coating Microparticles Separately with HIV Antibody and HIV Antigen Capture Reagents

In this example, the various analyte capture reagents are coated separately onto polystyrene microparticles (available from Seradyne Inc., Indianapolis, Indiana). Each of the reagents may be coated separately from each other or in various combinations with each other. After each of the analyte capture reagents is coated on their respective microparticles, the various coated microparticles are pooled together and used in the assay.

In the present example, the two monoclonal anti-HIV p24 antibodies (31-42-19 and 31-90-25) are coated together onto microparticles separate

from the microparticles coated simultaneously with recombinant HIV-1 and HIV-2 p41 antigens (pTB319 and pJC104). Although the exact amount may vary, in general, the coating procedure will approximate that described in Example 5. After blocking the physically separated microparticles with 10 mg/ml casein in 0.01 M Tris (pH 8.0), 0.15 M NaCl at 56°C for 18-24 hours, the microparticles again are washed as described in Example 5, pooled together, and diluted to a 0.015% suspension (wt/volume) in 0.05 M Tris 9 pH 8.0), 0.15 M NaCl, 1% BSA and 15% sucrose. At the pooling step, the microparticles may be pooled at various ratios to affect sensitivity and specificity of the assay in order to optimize their use.

Example 8

Simultaneous Detection of HIV Antibody and HIV Antigen on Microparticles Separately Coated With HIV Antibody and HIV Antigen Capture Reagents

The Abbott IM$_X$® Microparticle Enzyme Immunoassay (MEIA) system is used, although any system which employs microparticles can be used. The Abbott IM$_X$® MEIA system is thoroughly described in the Abbott IM$_X$® Operation and Customer Training Manuals (available from Abbott Diagnostic Division, Abbott Laboratories, Abbott Park, IL). In this assay, 100 µl of the 0.15% suspension prepared in Example 5 is mixed together with 100 µl of test sample suspected of containing HIV-1 and/or HIV-2 antibody and/or HIV-1 antigen, and incubated at 40°C for ten minutes in an Abbott IM$_X$® reaction cell to form a reaction mixture. HIV antibodies and/or HIV antigens bind to the microparticles in an antibody/antigen/microparticle complex. 150 µl of the reaction mixture is transferred onto a glass fiber matrix to which the microparticles are retained in an irreversible binding. The antibody/antigen/microparticle complexes then are reacted with 50µl of a probe consisting of biotinylated recombinant HIV-1 and HIV-2 recombinant p41 antigens (pTB319 and pJC104, previously described) and biotinylated F(Ab')$_2$ anti-HIV-1 p24 in 0.05M Tris 9 pH 8.0), 2% BSA, 0.25% saponin and 0.1% sodium azide at 40°C for ten minutes. 50 µl of an antibody conjugate consisting of goat anti-biotin alkaline phosphatase in 0.1 M Tris (pH 8.0), 0.5M NaCl, 0.9% Brij-35®, 1.0% BSA and 0.1% sodium azide then is allowed to react with the biotin probe/antibody/antigen/microparticle complexes for ten minutes at 40°C. Then, these microparticle complexes are washed six times with 0.05 M Tris (pH 8.0), 0.3 M NaCl and 0.1 % sodium azide, the biotin probe/antibody/antigen microparticle complex is reacted with

50 µl of the substrate methylumbelliferyl phosphase (MUP, Abbott Laboratories, Abbott Park, IL), and the fluorescence of the product , methylumbelliferon, (MU) is measured. The rate of MU production is proportional to the concentration of analyte(s) in the test sample.

It is contemplated that the assay of the invention can be optimized even further by varying assay conditions and/or incubation times, using various combinations of antigen or antibody capture or probe reagents, and other methods, reagents and conditions known to those skilled in the art. Thus, various other antibody capture reagents can be used, including HIV p24, gp120, gp160, p17, and others. The variance of the antibody capture reagent may then require the use of a different antigen capture reagent. All these variations are contemplated to be within the scope of this invention. Also, while some of the assays described in the examples used an automated system, it is well within the scope of the present invention that manual methods or other automated analyzers can be used or adapted to the assay of the present invention.

CLAIMS

WHAT IS CLAIMED IS:

1. An assay to simultaneously detect the presence, if any, of an HIV antigen analyte and/or an HIV antibody analyte which may be present in a test sample, comprising the steps of:

a) simultaneously or sequentially contacting the test sample with

i) at least one HIV antibody capture reagent which is specific for the HIV antibody analyte and which is attached to a solid phase,

ii) at least one HIV antigen capture reagent which is specific for the HIV antigen analyte and which is attached to a solid phase,

iii) an HIV antibody indicator reagent which comprises a member of a binding pair specific for the HIV antibody analyte labelled with a signal generating compound, and

iv) an HIV antigen indicator reagent which comprises a member of a binding pair specific for the HIV antigen analyte labelled with a signal generating compound,

thereby forming HIV antibody capture reagent/HIV antibody analyte/HIV antibody indicator reagent complexes and/or HIV antigen capture reagent/HIV antigen analyte/HIV antigen indicator reagent complexes; and

b) determining the presence, if any, of the HIV antibody analyte and/or the HIV antigen analyte in the test sample by detecting said signal generating compound associated with said complexes.

2. An assay to simultaneously detect the presence, if any, of an HIV antigen analyte and/or an HIV antibody analyte in a test sample, comprising the steps of:

a) simultaneously contacting the test sample with:

i) at least one HIV antibody capture reagent which is specific for the HIV antibody analyte and which is attached to a solid phase,

ii) at least one HIV antigen capture reagent which is specific for the HIV antigen analyte and which is attached to a solid phase, and

iii) an HIV antigen indicator reagent which comprises a member of a binding pair specific for said HIV antigen analyte labelled with a signal generating compound,

thereby forming a first mixture;

b) incubating said first mixture for a time and under conditions sufficient to form HIV antibody capture reagent/HIV antibody analyte complexes and HIV antigen capture reagent/HIV antigen analyte/HIV antigen indicator reagent complexes;

c) contacting said complexes with an HIV antibody indicator reagent which comprises a member of a binding pair specific for said HIV antibody analyte labelled with a signal generating compound, thereby forming a second mixture;

d) incubating said second mixture for a time and under conditions sufficient to form HIV antibody capture reagent/HIV antibody analyte/HIV antibody indicator reagent complexes; and

e) determining the presence, if any, of said HIV antigen analyte and/or said HIV antibody analyte in the test sample by detecting said signal generating compound associated with said complexes.

3. The assay of claim 1 or 2, wherein said HIV antibody capture reagent and said HIV antigen capture reagent are bound to the same solid phase.

4. The assay of claim 1 or 2, wherein said HIV antibody capture reagent is bound to a first solid phase and said HIV antigen capture reagent is bound to a different second solid phase.

5. The assay of claim 1 or 2, wherein said HIV antigen analyte is HIV p24 *gag* antigen and said HIV antigen capture reagent is anti-HIV p24 antibody selected from the group consisting of a monoclonal antibody, a polyclonal antibody, a recombinantly derived antibody, a monoclonal antibody fragment, a polyclonal antibody fragment and a fragment of a recombinantly derived antibody.

6. The assay of claim 5, wherein said monoclonal anti-HIV p24 antibody fragment and said polyclonal anti-HIV p24 fragment are $F(Ab')_2$ anti-HIV p24 antibody fragments.

7. The assay of claim 6, further comprising a hapten attached to said $F(Ab')_2$ fragment.

8. The assay of claim 5, wherein said anti-HIV p24 antibody is a mixture of monoclonal antibodies secreted by hybridoma cell lines ATCC Deposit Nos. HB 9725 and HB 9726.

9. The assay of claim 1 or 2, wherein said HIV antibody analyte is anti-HIV-1 antibody and/or anti-HIV-2 antibody and the HIV antibody capture reagent is a mixture of HIV-1 p41 *env* protein and HIV-2 p41 *env* protein.

10. The assay of claim 9, further comprising a hapten attached to said HIV-1 p41 *env* protein and said HIV-2 p41 *env* protein.

11. The assay of claim 10, wherein said HIV-1 p41 *env* protein and said HIV-2 *env* antigen are recombinantly or synthetically produced.

12. The assay of Claim 1 or 2, wherein said HIV antibody capture reagent includes at least one recombinant HIV-1 p41 antigen and at least one HIV-2 p41 antigen, and said HIV antigen capture reagent includes at least one anti-HIV p24 antibody.

13. The assay of Claim 12, wherein said anti-HIV p24 antibody is bound to a first solid phase and said recombinant HIV-1 p41 antigen and HIV-2 p41 antigen are bound to a different second solid phase.

14. An assay to simultaneously detect the presence, if any, of an HIV-1 p24 antigen, anti-HIV-1 p41 antibody and anti-HIV-2 p41 antibody in a test sample, comprising:

a) contacting the test sample with a solid phase to which at least one anti-HIV-1 p24 antibody, at least one recombinant HIV-1 p41 antigen and at least one HIV-2 p41 antigen have been attached, thereby forming a mixture;

b) incubating said mixture for a time and under conditions sufficient to form anti-HIV-1 p24 antibody/HIV-1 p24 antigen complexes and/or HIV-1 p41 antigen/anti-HIV-1 p41 antibody and/or HIV-2 p41 antigen/anti-HIV-2 p41 antibody complexes;

c) determining the presence of HIV-1 p24 antigen in the test sample by:

i) contacting said anti-HIV-1 p24 antibody/HIV-1 p24 antigen complexes with an anti-HIV-1 p24 antibody, capable of specifically binding HIV-1 p24 antigens, for a time and under conditions sufficient to form anti-HIV-1 p24 antibody/HIV-1 p24 antigen/anti-HIV-1 p24 antibody complexes,

ii) contacting said anti-HIV-1 p24 antibody/HIV-1 p24 antigen/anti-HIV-1 p24 antibody complexes with an indicator reagent comprising an anti-species antibody labeled with a signal generating compound, and

iii) detecting the signal generated as an indication of the presence of HIV-1 p24 antigen in the test sample; and

d) determining the presence of anti-HIV-1 p41 antibody and/or anti-HIV-2 p41 antibody in the test sample by:

i) contacting said HIV-1 p41 antigen/anti-HIV-1 p41 antibody complexes and/or HIV-2 p41 antigen/anti-HIV-2 antibody complexes with an indicator reagent comprising HIV-1 p41 antigen labeled with a signal generating compound and HIV-2 p41 antigen labeled with a signal generating compound, and

ii) detecting the signal generated as an indication of the presence of anti-HIV-1 p41 antibody and/or HIV-2 p41 antibody in the test sample.

15. An assay to simultaneously detect the presence, if any, of an HIV-1 p24 antigen and/or an anti-HIV-1 p41 antibody and/or anti-HIV-2 p41 antibody in a test sample, comprising:

a) contacting the test sample with a first solid phase to which at least one anti-HIV-1 p24 antibody has been attached and with a second solid phase to which recombinant HIV-1 p41 antigen and HIV-2 p41 antigen have been attached, to form a mixture;

b) incubating said mixture for a time and under conditions sufficient to form anti-HIV-1 p24 antibody/HIV-1 p24 antigen complexes and/or HIV-1 p41 antigen/anti-HIV-1 p41 antibody complexes and/or HIV-2 p41 antigen/anti-HIV-2 p41 complexes;

c) determining the presence of HIV p24 antigen in the test sample by:

i) contacting said anti-HIV-1 p24 antibody/HIV-1 p24 antigen complexes with an anti-HIV-1 p24 antibody capable of specifically binding HIV-1 p24 antigens for a time and under conditions sufficient to form anti-HIV-1 p24 antibody/HIV-1 p24 antigen/anti-HIV-1 p24 antibody complexes,

ii) contacting said anti-HIV-1 p24 antibody/HIV-1 p24 antigen/anti-HIV-1 p24 antibody complexes with an indicator reagent comprising an anti-species antibody labeled with a signal generating compound, and

iii) detecting the signal generated as an indication of the presence of HIV-1 p24 antigen in the test sample; and

d) determining the presence of anti-HIV-1 p41 antibody and/or HIV-2 p41 antibody in the test sample by:

i) contacting said HIV-1 p41 antigen/anti-HIV-1 p41 antibody complexes and/or said HIV-2 p41 antigen/anti-HIV-2 p41 antibody complexes with an indicator reagent comprising an HIV-1 p41 antigen labeled with a signal generating compound and an HIV-2 p41 antigen labeled with a signal generating compound; and

ii) detecting the signal generated as an indication of the presence of anti-HIV-1 p41 antibody and/or HIV-2 p41 antibody in the test sample.

16. The assay of Claims 14 or 15, wherein said anti-HIV-1 p24 antibody is a mixture of monoclonal antibodies secreted by hybridoma cell lines ATCC Deposit Nos. HB 9725 and HB 9726.

17. A test kit for simultaneously detecting HIV antigen analyte and/or HIV antibody analyte in a test sample, comprising:

a) at least one solid phase with an attached HIV antigen capture reagent comprising at least one anti-HIV p24 antibody and an attached HIV antibody capture reagent comprising at least one HIV p41 antigen;

b) an indicator reagent for the HIV antigen analyte comprising a member of a binding pair which is specific for the HIV antigen analyte and which is labelled with a signal generating compound; and

c) an indicator reagent for the HIV antibody analyte comprising a member of a binding pair which is specific for HIV antibody analyte and which is labelled with a signal generating compound.

18. The test kit of Claim 17, wherein said HIV antigen capture reagent comprises at least one anti-HIV p24 antibody attached to a first solid phase, and said HIV antibody capture reagent comprises at least one HIV p41 antigen attached to a second solid phase.

INTERNATIONAL SEARCH REPORT

Inte..ational application No.
PCT/US93/03224

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12Q 1/70
US CL :435/5, 7.1; 436/820
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/5, 7.1; 436/820

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Acquired Immune Deficiency Syndrome, Vol. 1, No.3, issued 1988, Haseltine, "Replication and Pathogenesis of the AIDS virus", pages 217-240, see entire document.	1-18
Y	Science, Vol. 233, issued 18 July 1986, Clavel et al., "Isolation of a New Human Retrovirus from West African Patients with AIDS", pages 343-346, see entire document.	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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